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Expression of rice *OsMyb*4 transcription factor improves tolerance to copper or zinc in canola plants

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Abstract

The effects of copper and zinc salts on transgenic canola plants expressing rice transcription factor (TF) OsMYB4 were investigated. Transgenic plants (TPs), which showed a high *OsMyb*4 expression in response to either Cu or to Zn excess, were used for the current study. In leaves of TPs, the content of Cu was equal and the content of Zn was significantly higher than in non-transformed plants (NTPs). The TPs grown on an extremely high concentration of heavy metals (HMs; 150 μM $CuSO_4$ or 5 000 μM $ZnSO_4$) were able to survive for more than 15 d, while NTPs died after 7 - 9 d of incubation. This indicates that expression of *OsMyb*4 in canola plants improved their HM tolerance. The TPs tolerance to HMs was confirmed by a higher shoot biomass than that in NTPs. Excess of HMs caused oxidative stress (indicated by increase in malondialdehyde content) especially in leaves of NTPs. This data suggests a protective role of the *OsMyb*4 TF in oxidative stress. The HMs caused a lower decrease in activities of superoxide dismutase and guaiacol peroxidase in TPs than in NTPs. Higher tolerance of TPs to HMs was also suggested by a considerable increase in the content of low-molecular phenolic compounds, including flavonoids and anthocyanins, as well as proline (a potential antioxidant and chaperone). These data suggest that OsMYB4 may play a role as a positive regulator of phenylpropanoid pathway and proline synthesis. The created canola *OsMyb*4 TPs may be useful for future applications in phytoremediation of HM-polluted soils.

Additional key words: anthocyanins, *Brassica napus,* guaiacol peroxidase, heavy metals, malondialdehyde, proline, superoxide dismutase.

Introduction

Improvement of plant tolerance to excessive concentrations of heavy metals (HMs) is an important task since the soils are polluted by HM not only in the areas of mining and processing, but also in other numerous industrial regions. Furthermore, HMs pollution of agricultural soils is worsening as a result of long-term application of fertilizers and chemicals for plant protection that contain HMs as impurities (Kabata-Pendias 2010). Soil contamination by HMs is a strong stress factor for plants. Excess of HM results in growth retardation, reduction in the photosynthetic pigments, disturbance of the primary and secondary metabolism, and development of oxidative stress (Changela *et al*. 2003, Yruela 2009). Due to the ability to form bonds with organic molecules, Cu ions occupy the number one position in Irving-Williams series of toxicity among HMs, being directly involved in the cellular redox reactions (Yruela 2009). Zinc ions also cause oxidative stress through indirect mechanisms such as interaction with the antioxidant defense system, disruption of the electron transport chain, and induction of lipid peroxidation (Hossain *et al*. 2012). Adverse effects of HM ions are also caused by their competition with essential ions in uptake into the plants and in translocation and redistribution between plant organs (Ivanova *et al*. 2011).

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Abbreviations: CAT - catalase; HM(s) - heavy metal(s); LMPC - low molecular phenolic compound; MDA - malondialdehyde; MYB - myeloblastosis protein family; NTP(s) - non-transformed plant(s); POD - guaiacol peroxidase; ROS - reactive oxygen species; SOD - superoxide dismutase; TBA - thiobarbituric acid; TF(s) - transcription factor(s); TP(s) - transgenic plant(s).
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Excess of HMs also exerts an indirect toxic effect manifested by decrease in extension growth due to reduction of tissue hydration and transpiration (Kholodova *et al*. 2011). Another indirect toxic effect comprises the inactivation of enzymes due to binding HMs to cysteine residues leading to misfolding proteins and inhibition of enzyme activity (Dal Corso *et al*. 2008). Thus, exposure of plants to high concentrations of HMs triggers a wide range of physiological and metabolic alterations (Nagajyoti *et al*. 2010).

Transcription factors (TFs) appear to be the first line in the plant response to various external stressors including HMs (Lata *et al*. 2011). They play an important role by controlling the genes responsible for HM uptake, transport, and processes of HM detoxification. The members of myeloblastosis protein family (MYB) of TFs participate in plant growth regulation as well as in protection against different stressors (Singh *et al*. 2002). The properties of MYB4 TF attracted special attention of researchers; and R2-R3 *Myb4*-like genes were identified in a number of plant species (Du *et al.* 2009, Park *et al*. 2010, Geethalakshmi *et al*. 2015). Baldoni *et al.* (2013) have performed an *in silico* analysis to identify putative OsMYB4-like proteins in several dicot and monocot species in addition to rice. The created phylogenetic tree showed that in rice, OsMYB4 belongs to a small subfamily that also contains two additional members. In monocots, three subgroups corresponding to the three rice MYB factors were found whereas in dicots, this three group subfamilies did not show a clear subgroup organization. In addition, some closely related MYB factors, from both monocot and dicot species, cluster independently to the OsMYB4-like proteins (Baldony *et al*. 2013).

It was shown that overexpression of the *A. thaliana AtMyb4* gene improves tolerance to UV-B radiation *(*Jin *et al*. 2000). Akagi *et al*. (2009) showed that the *DkMyb4* gene from *Diospyros kaki* activates genes required for proanthocyanidin biosynthesis. The *OsMyb*4 gene from rice has been introduced into several plant species and its role in abiotic/biotic stress responses has been evaluated (Pandolfi *et al*. 1997, Vannini *et al*. 2004, 2006, 2007, Mattana *et al.* 2005, Pasquali *et al.* 2008, Laura *et al.* 2010*,* Gomaa *et al*. 2012, Soltész *et al*. 2012, Docimo *et al.* 2013, Aydin *et al.* 2014).

Creating transgenic plants represents an important approach to improve plant tolerance, and it also offers an excellent opportunity to investigate the mechanisms of stress adaptation. In the current study, we transformed canola plants with a construct containing the *OsMyb*4 gene expression cassette. Excess concentrations of Cu and Zn salts were used as stress agents to test whether *OsMyb*4 may improve the tolerance of transgenic canola plants to HM stress, and if so, to find out physiological and metabolic changes responsible for the resistant phenotype.

Materials and methods

Plants and growth conditions: Experiments were performed using spring canola (*Brassica napus* L. cv. Westar) plants (NTPs) and the same cultivar transformed with the transcription factor *OsMyb*4 gene (TPs). The cotyledons were transformed by *Agrobacterium tumefaciens* strain AGL0 according to the procedure described by Malyshenko *et al*. (2003). The expression cassette COR15aOsMyb4 used for canola transformation was described in Vannini *et al.* (2004), Pasquali *et al.* (2008), and Aydin *et al.* (2014). This cassette contained the *OsMyb*4 full length cDNA (accession number Y11414), driven by *A. thaliana COR15a* stress-inducible promoter, and the *nos* terminator. The expression construct also included a kanamycin marker for selection in *E. coli*, and a *npt*II marker (under control of *nos* promoter and terminator) for plant selection. Resulting canola TPs were propagated by cuttings and after rooting on the Murashige and Skoog (1962) agar medium (MS), young plants were set into soil to obtain the seeds of the 1st (T1) generation. The young plants from these seeds were tested for the presence of the inserted *OsMyb*4 gene by PCR (see DNA extraction and PCR section below). The positively tested seedlings were transferred to low temperature (+4 °C) to induce *OsMyb*4 transcription. Plants with high *Myb*4 gene expression were grown to obtain the seeds of the T2 generation using the procedure described above.

From the seeds of T2 plants, the selected TPs were maintained under *in vitro* conditions. For the experiments, the selected lines of TPs (as well as NTPs) were propagated *in vitro* by cuttings and after rooting the plants were cultivated on liquid Hoagland and Snyder (1933) medium containing 0.25 μM $CuSO_4$ and 1 μM $ZnSO_4$. Plants (one plant in 800-cm^3 vessel) were grown under a 12-h photoperiod, an irradiance of 250 μmol m^{-2} s^{-1} (400 W, *JANLUX MH-GT* lamps*, Intersvet*, Moscow, Russia), and day/night temperatures of 20 - 22/17 - 19 °C for 4 to 5 weeks. The medium was changed weekly.

To evaluate gene expression and for other tests, plants with 5 - 6 leaves were exposed to HM stress for 15 d. HMs were added to the nutrient medium as $CuSO_4$ (25 - 150 μM) or $ZnSO_4$ (500 - 5 000 μM). For biochemical and molecular analyses, the 3rd and 4th leaves were harvested, cutted to approximently 2 × 2 cm pieces with a scalpel, the resulting pieces were piled together and mixed. Biomass samples (200 mg) were frozen in liquid nitrogen and stored at -80 °C.

DNA extraction and PCR: The DNA from T2 plants was isolated by the method of Fulton *et al*. (1995). DNA

quality was assessed by the 1 % (m/v) agarose gel electrophoresis. The PCR analysis was performed to detect the *OsMyb*4 gene in TPs using specific primers: forward primer 5'-CGGGAGGACGGACAACGAG-3' and reverse primer 5'-GGATGGCGGCGCGACGAAC-3' (Gomaa *et al*. 2012) and *Taq* polymerase kit from *Eurogen* (Moscow, Russia). PCR was performed with the following program: 94 °C for 2 min and then 30 cycles of denaturation at 92 °C for 1 min, annealing at 62 °C for 1 min, DNA extension at 70 °C for 1 min, and final extension at 70 °C for 10 min. The size of amplicon was 484 bp. To confirm the presence of the *nptII* gene in TPs, a pair of primers, (forward 5'-GTGGAGAGGCTA TTCGGCTA-3' and reverse 5'-CCACCATGATAT TCGGCAAG-3') were used in a PCR using the program described above for the *OsMyb4* gene with the exception of Tm (58 °C instead of 62 °C).

RNA extraction and semi-quantitative (sq) reverse transcrition (RT)-PCR: RNA was extracted from leaves using *TRIzol1* reagent (*Invitrogen*, Carlsbad, USA) according to the manufacturer′s instructions. The RNA was treated with RNase-free DNase I (*Fermentas*, Vilnius, Lithuania) to remove any genomic DNA contamination before reverse transcription reaction. First cDNA strands were synthesized using 5 μg of total RNA with reverse transcriptase *RevertAid* (*Fermentas*) according to supplier's instructions. For *OsMyb*4, RT-sqPCR was performed using specific primers and conditions, described above. The cDNA fragment generated from 18S ribosomal RNA was used as a reference (forward primer: 5'-GAGTGATGTGCCAGA CCTAGGAATT-3'; reverse primer: 5'-ATGCTGATC CGCGATTACTAGC-3'). The samples were subjected to 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 1 min, extension at 70 °C for 1 min, and a final extension at 70 °C for 10 min. The amplification products were separated by 1 % agarose gel electrophoresis.

Plant treatments and following analyses: For physiological and biochemical evaluations nine different plant groups were used: control NTPs without or with HM addition (100 μM $CuSO_4$ or 3 000 μM $ZnSO_4$), the TPs line 6 (TP6) without/with Cu addition (100 μM $CuSO_4$), and TPs line 5 (TP5) without/with Zn addition (3 000 μM $ZnSO_4$). Exposure to HMs lasted 15 d.

Fresh mass (f.m.) of 5 - 6 plants was measured before the initiation of the HM treatments (the start point) and after 15 d (the final point). The average daily specific increment of biomass ($Bm_{(incr)}$) was calculated according to the formula: $Bm_{(incr)} = (Bmf - Bms)/(Bms \times 15)$, where Bmf was plant biomass at the final time of the experiment and Bms the biomass at the start point.

Biomass accumulation or average daily specific increment was the ratio between plant f.m. at 15th day and the f.m. at the start point calculated per 1 d.

For biochemical analyses, the parts of fully expanded leaves were immediately frozen in liquid nitrogen at the end of the experimental period and stored at -80 °C. The remaining parts of leaves were dried at 70 °C to a constant mass and used for determination of HMs content.

Dried leaf samples (50 mg) were treated with the mixture of concentrated nitric (1.5 cm^3) and perchloric (0.6 cm^3) acids overnight, heated at 180 °C for 2 h, and after cooling to room temperature, 0.05 cm^3 of 30 % (v/v) H_2O_2 was added. After dilution to 10 cm^3 with distilled water, Cu and Zn concentrations were measured with *AAS-FM 400* atomic-absorption spectrometer (*Labist*, Moscow, Russia).

The lipid peroxidation was determined by measuring the malondialdehyde (MDA) content according to Heath and Packer (1978).

For determination of superoxide dismutase (SOD, EC 1.15.1.1) and peroxidase (POD, EC 1.11.1.7) activities, leaf samples (500 mg) were homogenized in 3 cm^3 of ice-cold 50 mM Na-phosphate buffer (pH 7.8) containing 2 % (m/v) polyvinylpyrrolidone. After centrifugation at 10 000 *g* and 4 °C for 30 min, the supernatants were used in SOD and POD assays. POD activity was determined according to Ridge and Osborne (1971). One unit (U) of POD activity was defined as the amount of the enzyme that caused a change of 0.001 in absorbance per min. SOD activity was evaluated according to Beauchamp and Fridovich (1971). One U of SOD activity was defined as the amount of enzyme causing 50 % inhibition of photochemical reduction of NBT. The protein content in plant extracts was estimated with Coomassie R-250 solution according to Esen (1978).

The free proline content in leaf samples (100 - 200 mg) was measured with an acidic ninhydrin reagent method according to Bates *et al*. (1973).

Total content of low molecular phenolic compounds (LMPCs) was evaluated using Folin-Denis reagent as described in Zagoskina *et al*. (2003). Flavonoid content was measured in the 96 % (v/v) ethanol leaf extracts by adding $AlCl_3$ solution and recording absorbance at 415 nm (Gage and Wendei 1950) using *Genesis 20* spectrophotometer (*ThermoFisher Scientific*, Madison, WI, USA). Rutin was used as reference standard. Anthocyanin content was evaluated in leaf extracts prepared using the extraction buffer containing 3 M HCl, H_2O, and MeOH (1:3:16, v/v/v). Absorbances at 530 and 653 nm were then read and the content of anthocyanis was calculated as $A_{530} - 0.24 \times A_{653}$ according to Murrey and Hackett (1991), based on the comparison with cyanidin absorbance.

Statistical analyses: Three identical experiments using at least 5 plants for each experiment were analyzed with one-way *ANOVA* using the statistical program *SPSS v. 9*. To evaluate the difference between TPs and NTPs the Student's *t*-test was performed and $P \leq 0.05$ was considered as statistically significant.

Results

The presence of the *OsMyb*4 and *npt*II genes in transformed seedlings was checked by PCR analysis. *OsMyb*4 was present in the genomes of 40 out of 50 analyzed seedlings, the selection marker *npt*II was found in 35 seedlings, and 25 seedlings contained both *npt*II and *OsMyb*4. The transformants were propagated *in vitro* by cuttings, the clones were planted on the standard nutrient solution supplemented with either 3 000 μM $ZnSO_4$ or 100 μM $CuSO_4$. RT-PCR analysis of 15-d-old plants showed that only 7 out of the 25 transformants expressed the *OsMyb*4 gene. Although each line was tested for growth under elevated Zn or Cu, the *OsMyb*4 transgene was induced in five lines only by toxic concentrations of Zn (lines TP1 - TP5), and in remaining two lines only by toxic concentrations of Cu (lines TP6 and TP7, data not shown).

For further investigation, two lines that showed high *OsMyb*4 expression were chosen: TP5 for $ZnSO_4$ and TP6 for $CuSO_4$. After propagation *in vitro* and rooting, the plants were transferred to nutrient medium supplemented with different concentrations of $ZnSO_4$ or $CuSO_4$. On the 15th day, we have found that *OsMyb4* was not expressed in the leaves of plants grown at relatively low HM concentrations (Fig. 1). The *OsMyb*4 expression was only observed at 50 - 150 μM of $CuSO_4$ or 2 000 - 5 000 μM of $ZnSO_4$ (Fig. 1*A*,*B*). Therefore, the consequent experiments were performed with plants exposed to 100 μM of $CuSO_4$ (TP6) and 3 000 μM of $ZnSO_4$ (TP5) for 15 d.

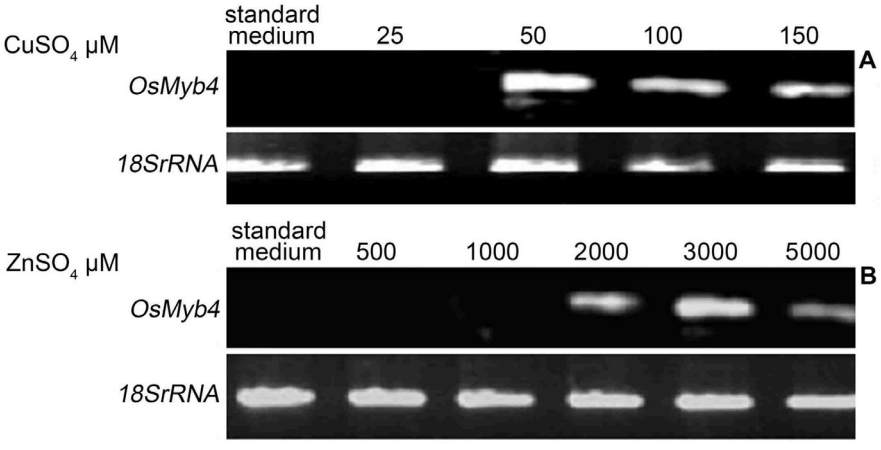

Fig. 1. Effect of various concentrations of $CuSO_4$ on *OsMyb4* gene expression in line TP6 (*A*) or $ZnSO_4$ on *OsMyb4* gene expression in line TP5 (*B*). Leaves of transgenic canola plants grown on standard medium containing 0.25 μM $CuSO_4$ and 1 μM $ZnSO_4$ or plants exposed to $CuSO_4$ (25 - 150 μM) or $ZnSO_4$ (500 - 5000 μM) for 15 d were used for total RNA isolations. cDNA was amplified with the *OsMyb4* or *18S rRNA* genes specific primers and resulting fragments were separated on a 1 % agarose gel.

Next, we determined which HM concentrations were able to kill the plants after 15 d of growth. We have found that NTPs grown on 150 μM $CuSO_4$ or 5 000 μM $ZnSO_4$ died after 7 - 9 d, whereas TPs survived more than 15 d under the same conditions (Fig 2). Thus, the expression of the OsMYB4 protein in canola plants increased their resistance to severe HM stress. On lower concentrations of HMs, however, both the types of plants were able to survive and grow after 15 d.

A further criterion of plant response to HMs was the increment of fresh mass (*i.e.*, an average increase in f.m. per day). The average increase of f.m. for control plants was somewhat higher than that of TPs (Fig. 2), however, for TP5 this difference was not statistically significant.

When either Cu or Zn at high, but permissive concentration were included in the nutrient medium, the great reduction of biomass as well as some decrease in biomass increments were observed for all plants. The effect of Cu was higher than of Zn (Fig. 2 and data not shown). Furthermore, the reduction of growth (judged by decrease in biomass increment) was less pronounced for canola TPs than for the NTPs. For instance, 100 μM $CuSO_4$ inhibited growth of TP6 by app. 60 % with respect to the control TPs, whereas growth of NTPs was reduced by app. 70 %; however, the difference observed between NTP and TP6 was not statistically significant. In the presence of 3 000 μM $ZnSO_4$, the f.m. was reduced by about 38 and 64 % for TP5 and NTPs, respectively in comparison to the controls. The daily increment of f.m. of canola TP5 exposed to Zn was significantly higher (at $P \leq 0.05$) than that of the NTPs (Fig. 2 and data not shown).

Additionally, it was found that both types of canola plants growing on a standard medium showed very similar content of HMs in leaves (Table 1). After treatment with 100 μM $CuSO_4$ for 15 d, the content of Cu in the NTP and TP6 leaves similarly increased. Accumulation of Zn in the plants grown on 3 000 μM $ZnSO_4$ was much more pronounced: the content of Zn in the leaves of TP5 showed the increase (up to 33.5 %) in

comparison to NTPs (Table 1). The data described in Table 1 were statistically analyzed and this analysis showed that all the differences in TM content between TPs and corresponding control NTPs were insignificant, with the exception of Zn accumulation in TP5, where Zn levels were higher than in control NTPs, and the differences were statistically significant. Statistical analysis also indicated that despite the fact that TP5 plants accumulate Cu and Zn to somewhat higher levels than TP6, these differences were not significant. One explanation that this accumulation difference trend takes place could be the pleiotropy of plant transformation.

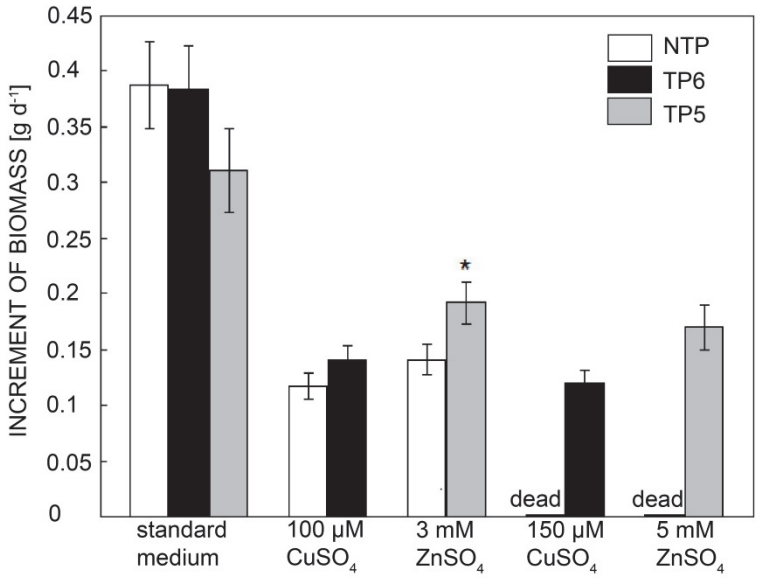

Fig. 2. Effect of Cu or Zn on growth of NTPs and TPs expressing the *OsMyb4* gene. Plants were grown on Hoagland-Snyder standard medium containing 0.25 μM $CuSO_4$ and 1 μM $ZnSO_4$ or standard medium supplemented either with 100 and 150 μM $CuSO_4$ or 3 and 5 mM $ZnSO_4$ for 15 d. Increment of biomass was calculated as the ratio between plant f.m. at 15th day and the f.m. at the start point per 1 d. Means ± SEs, $n = 3$; the *asterisk* marks a statistically significant difference at 5 % level.

Table 1. Content of Cu and Zn in canola NTPs and TPs (expressing the *OsMyb*4 gene) plants when grown for 15 d on control Hoagland-Snyder standard medium or those containing 100 μM $CuSO_4$ or 3 000 μM $ZnSO_4$. Means ± SEs, $n = 3$, means followed by different lowercase letters are statistically different at $P < 0.05$ according to Student's *t*-test.

Lines	Control		$CuSO_4$		$ZnSO_4$	
	Cu [μg g^{-1}(d.m.)]	Zn [μg g^{-1}(d.m.)]	Cu [μg g^{-1}(d.m.)]	[% of NTP]	Zn [μg g^{-1}(d.m.)]	[% of NTP]
NTP	9.87 ± 3.12a	35.2 ± 4.10a	88.7 ± 9.06a	100	36.3 ± 1.82a	100
TP6	11.72 ± 1.11a	31.7 ± 4.88a	98.5 ± 6.77a	111.0	43.7 ± 1.12a	120.6
TP5	13.02 ± 2.67a	40.6 ± 2.17a	99.5 ± 8.24a	112.2	48.4 ± 0.87b	133.5

Accumulation of reactive oxygen species (ROS) is a typical response to HMs, which is particularly characteristic for Cu. Furthermore, lipid peroxidation is induced under oxidative stress. In canola plants grown on standard medium, the content of MDA was low and did not differ significantly among NTPs and TPs (Fig. 3*A*). The MDA content sharply increased in NTPs exposed to $CuSO_4$, whereas in TPs, the MDA increase was much lower. The effect of $ZnSO_4$ on MDA accumulation was less pronounced than that of Cu and the difference between NTPs and TPs was not statistically significant (Fig. 3*A*).

The activities of ROS degrading enzymes are usually induced by oxidative stress, however, in our experiments the SOD activity in NTPs treated with 100 μM $CuSO_4$ decreased more than twice when compared to plants growing on standard medium, while the SOD activity in TPs grown on Cu-containing medium did not change (Fig. 3*B*). No significant differences in SOD activity were detected between NTPs and TPs grown on $ZnSO_4$.

The negative effect of both HMs was even more evident for the POD activities, which were considerably reduced in comparison with the untreated controls. Moreover, the decreases in POD activities were less pronounced in TPs than in NTPs (Fig. 3*C*).

In response to stresses, plants often synthesize soluble phenolic compounds and proline. Therefore, the content of LMPCs, flavonoids, anthocyanins, and free proline

was examined in HM-treated plants. On a standard medium, the content of these compounds did not differ between NTPs and TPs. However, HM stress has induced the accumulation of low molecular antioxidants, especially in TPs (Fig. 4). For example, upon exposure to Cu or Zn, the total content of LMPCs in TPs was 1.4-times higher than that in NTPs (Fig. 4*A*). As concern flavonoids and anthocyanins, the increase was up to 2.5-times higher for TPs relatively to the NTPs (Fig. 4*B*,*C*). The free proline content in canola leaves was relatively low with no difference between NTPs and TPs. Upon HMs treatments, the proline content markedly increased up to 15-times at $ZnSO_4$ and even up to 27-times at $CuSO_4$ (Fig. 4*D*). Moreover, the proline content was significantly higher in HM stress exposed TPs compared to NTPs (Fig. 4*D*).

Discussion

We introduced the *OsMyb*4 gene under a stress-inducible COR15a promoter into canola plants and showed that the transgene was expressed only under high concentrations of HMs (Fig. 1). Thus, it appears that COR15a cold-inducible promoter may act as a heavy metals-responsive as well. To our knowledge, it is the first report describing that this promoter is regulated by HMs.

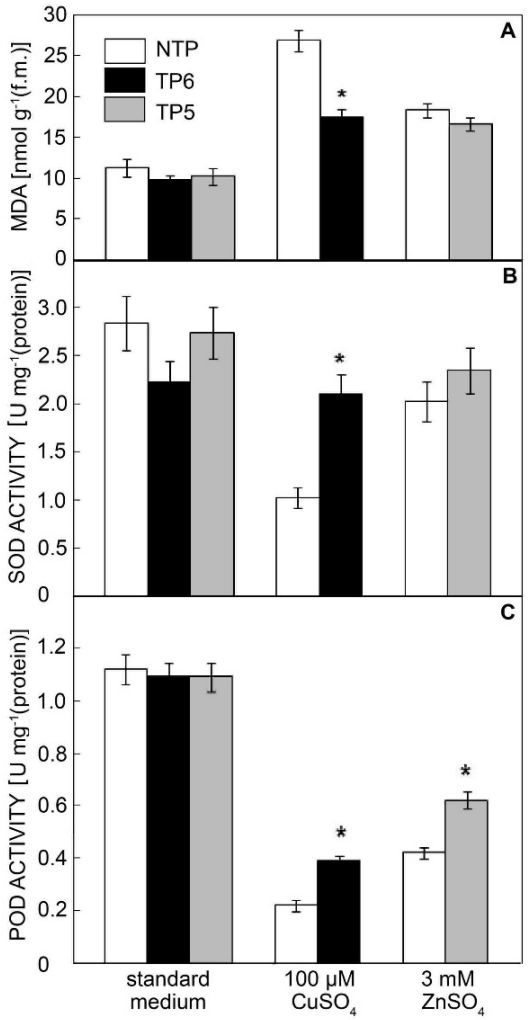

Fig. 3. Effect of HMs on MDA content (*A*), superoxide dismutase activity (*B*), and guiacol peroxidase activity (*C*) in NTPs and TPs expressing the *OsMyb4* gene. Plants were grown on Hoagland-Snyder standard medium containing 0.25 μM $CuSO_4$ and 1 μM $ZnSO_4$ or standard medium supplemented either with 100 μM $CuSO_4$ or 3 mM $ZnSO_4$ for 15 d. Means ± SEs, $n = 3$; *asterisks* mark significant differences at 5 % level.

When canola plants were treated with the highest concentrations of HMs, *OsMyb*4-expressing transformants had clear advantage; they continued to grow during at least 15 d whereas all NTPs have died within one week. This result allowed us to conclude that expression of the *OsMyb*4 gene in canola increased plant tolerance to HMs. In addition, a more noticeable delay in growth of NTPs than of TPs was seen when plants were treated with 100 μM $CuSO_4$ or 3 000 μM $ZnSO_4$.

Plant response to HM stress consists of a number of common reactions as well as a set of strictly specific responses depending on the nature of HM (Yruela 2009, Kumar *et al*. 2015). The metal-responsive transcription factor 1 (MTF-1) serves as the sensor for Zn (Rutherford and Bird 2004, Günther *et al*. 2012, Hardyman *et al*. 2016), whereas a copper-dependent transcription factor (ACE-1) plays a similar role in Cu sensing (Keller *et al*. 2005, Saijo and Nagasawa 2014, 2015). It was shown in our study that the expression of the *OsMyb*4 gene in TPs was induced by only one of the HMs used. We could not identify transgenic lines responding by *OsMyb*4 gene expression to Cu as well as to Zn simultaneously. Apparently, the independent activation pathways of the *OsMyb*4 gene expression by Cu or by Zn ions due to the specificity of their sensors were the basis for this phenomenon. Perhaps, the plant transformation caused some kind of malfunction in Cu and Zn signal transduction pathways upstream of the *OsMyb*4 target gene (or in the gene itself) in TP5 and TP6, respectively. However, we should note that the *OsMyb*4 was induced in both types of transgenic plants under cold stress (data not shown).

In addition, in eukaryotes, the accessibility of DNA specific binding sites for individual sensors plays an important role in regulation of gene expression. The availability of these sites may be altered by the "interception" of the receptor protein molecules by numerous non-specific low affinity DNA binding sites. This, in-turn, often causes a decrease in intensity of target transgene expression in eukaryotes (Smith and Matthews 2016), shown in our study as well (Fig. 1).

One possible reason for better performance of our TPs

under HM stress could be the restriction of HM uptake or HM translocation from roots to shoots. Indeed, Cu-exposed rice plants show 30 - 40 % decrease of the Cu content in leaves when they are pretreated with salicylic acid and so toxic effects are reduced (Mostofa and Fujita 2013). However, in canola TPs, Zn was higher than in NTPs and Cu content similar in both TPs and NTPs, suggesting that HM uptake and translocation was not negatively affected by *OsMyb*4 expression (Table 1). One of the possible explanation of this finding could be the pleiotropic effect of *OsMyb*4 transformation on canola plants. The transformation may lead to an increase in efficiency of their transport into the root cells or their transport to shoots or both.

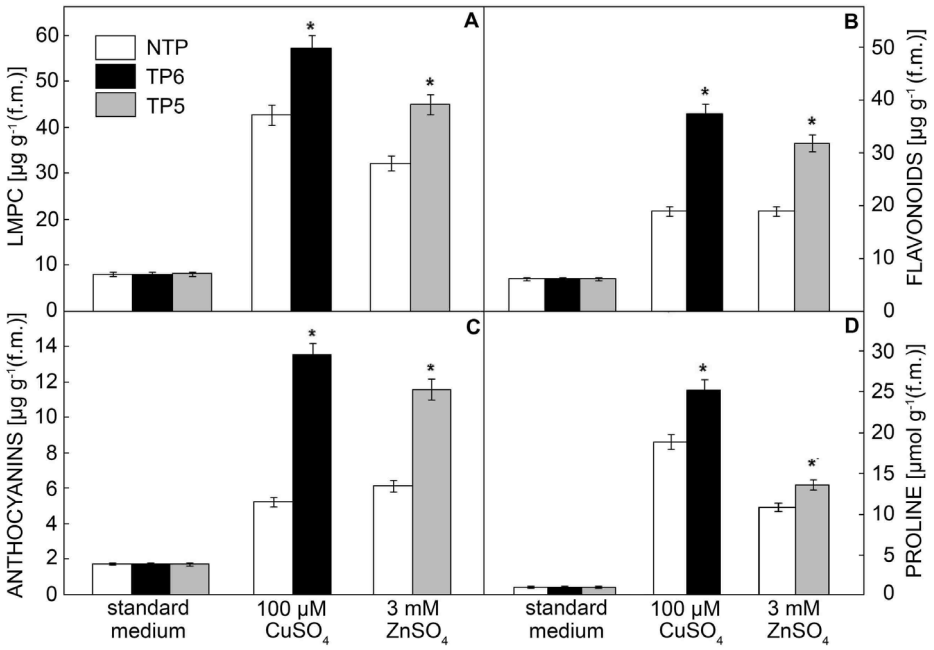

Fig. 4. Effect of HMs on content of low molecular phenolic compounds (LMPCs; *A*), flavonoids (*B*), anthocyanins (*C*), and free proline (*D*) in NTP, TP5, and TP6 canola plants. Plants were grown on Hoagland-Snyder standard medium containing 0.25 µM $CuSO_4$ and 1 µM $ZnSO_4$ or standard medium supplemented either with 100 µM $CuSO_4$ or 3 mM $ZnSO_4$ for 15 d. Means ± SEs, $n = 3$; *asterisks* mark significant differences at 5 % level.

Treatments with HMs considerably elevate ROS production, resulting in oxidative stress (Kreslavski *et al*. 2012). The MDA content is significantly reduced in tobacco plants transformed with the *LbDREB* gene when compared to NTPs under severe Cu stress (Ban *et al*. 2011). In our experiments, peroxidation of lipids was lower in canola TPs in comparison with NTPs upon Cu stress (Fig. 3*A*). These data are in accordance with the well-known role that Cu plays as a redox-active transition metal in plants (Yruela 2013). Lower MDA content under Zn stress may show that Zn has some influence on oxidative stress through indirect mechanisms (Hossain *et al*. 2012).

Usually, oxidative stress is accompanied by an increase in antioxidant enzyme activities as well as by stimulation of the expression of corresponding genes (Kreslavski *et al*. 2012). However, in this study, the decrease in SOD and POD activities was seen on HM media (Fig. 3*B*,*C*). A decrease in the antioxidant activities in response to Zn was earlier reported for rapeseed plants (Wang *et al*. 2009). Further, the CAT activity decreases in leaves of rice exposed to $CuSO_4$, while the MDA content is significantly increased (Mostofa and Fujita 2013). In addition, a decrease in CAT activity has been reported in *Pistia stratiotes* grown under severe Cu stress (Upadhyay and Panda 2009). In radish seedlings, both CAT and SOD activities are decreased under Cu treatment (Lukatkin *et al*. 2014). In our experiments, reduction of SOD, and especially of POD activities under Cu and Zn treatments (Fig. 3) may be caused by the relatively long exposure to HM (15 d). Similarly, in plants of two *Malus* species, CAT and POD activities strongly increase during the first 3 d under drought stress, however, these activities decrease to a standard level after 6-d drought (Li *et al*. 2014).

Proline, an iminoacid, is a well-known compatible osmolyte with antioxidant properties, which participates in plant defense systems against different abiotic stressors (Kuznetsov and Shevyakova 1999, Hossain *et al*. 2014). In addition, free proline is also a chaperone that can protect and restore the tertiary structure of protein molecules, which could be damaged under severe stresses (Szabados and Savoure 2011). Probably, the multi-functionality of free proline, and above all, its role as a chaperone, determined proline increase up to 15 - 27-fold under Cu or Zn stress when compared to its control level,

especially in TPs (Fig. 4*D*).

The role of the *OsMyb*4 gene in the synthesis of phenolic compounds as an important class of antioxidant substances has been accessed by Vannini *et al*. (2006). It has been found that *A. thaliana* plants expressing the OsMYB4 protein show activation of genes belonging to the phenylpropanoid pathways that are involved in the synthesis of flavonoids and anthocyanins. In addition, Gomaa *et al*. (2012) have found that canola TPs expressing the *OsMyb*4 gene are more tolerant to exposure to low temperatures and accumulated more LMPCs, including anthocyanins, than NTPs.

Our data suggest that transgenic canola plants expressing *OsMyb*4 performed better under HM stress because the rice OsMYB4 protein promoted the accumulation of proline and soluble phenylpropanoid compounds, especially under Cu stress (Fig. 4). Previously, participation of the OsMYB4 TF in protection against stresses has been shown for transgenic rice (Park *et al*. 2010), where this protein is over-expressed under control of the constitutive CaMV35S promoter. The authors have demonstrated that the OsMYB4 controls a hierarchical network of TFs that regulate the expression of several regulatory sub-clusters of genes associated with cellular defense. A large group of genes participating in the synthesis of phenylpropanoid and isoprenoid compounds that are involved in antioxidant defense responses has been activated in rice plants under over-expression of *OsMYB4* gene.

Uptake and distribution of HMs do not appear to be negatively affected by *OsMyb*4 expression in our TPs. The protection functions of the OsMYB4 protein are particularly visible under long-term exposure to severe HM stress, which reflects the new state of canola plants acclimated to high concentrations of HMs (Kuznetsov 1993). In this study we attempted to unravel the mechanisms of increased HM tolerance in transgenic canola plants without a decrease in Cu content (TP6) or even with increase in Zn content (TP5). Our findings suggest that increased HM tolerance of transgenic plants may be linked to the ability of plants to actively accumulate LMPCs with strong antioxidant properties (Fig. 4). A direct evidence in support of this conclusion has been reported by Park *et al*. (2010). They have shown that in rice, the *OsMyb*4 gene activates the expression of a set of genes involved in phenolic compound synthesis. There might be another reason for this phenomenon – more effective HM ion sequestration system functioning from cytoplasm to the central vacuole. This hypothesis, however, requires experimental confirmation, which was not a goal of the current study.

In the future, we are planning to study the global canola plant response to the HM stress on the transcriptional level and the mechanism(s) by which the *OsMyb*4 transgene expression leads to the improved tolerance to Cu or Zn toxicity observed in our TPs. The properties described above make the canola *OsMyb*4 TPs suitable for profound investigations of HM tolerance mechanisms as well as for future agricultural applications for both Cu and especially Zn phytoremediation of HM-polluted soils.

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